Benzodiazepines and Metabolites from Blood and Urine by LC/MS (SRM)

1 Introduction

Benzodiazepines are one of the most commonly prescribed classes of drugs in the United States. They are also frequently abused. Therapeutic blood concentrations vary for benzodiazepines, depending on whether they are considered high dose benzodiazepines (e.g., diazepam) or low dose benzodiazepines (e.g., triazolam). Benzodiazepines are usually excreted into the urine as glucuronide metabolites, and may persist in the urine for days after administration due to elimination half lives that may exceed 24 hours.

2 Scope

This procedure allows for the screening and confirmation of the following benzodiazepines in blood and urine: 7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxymidazolam, α-hydroxytriazolam, alprazolam, chlordiazepoxide, clonazepam, desalkylflurazepam, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, n-desmethylflunitrazepam, nordiazepam, oxazepam, temazepam, and triazolam. It also provides for quantitation of these compounds in blood. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Biological specimens are qualitatively assayed and/or quantified for benzodiazepines and their metabolites. Specimens are mixed with deuterated internal standards. Proteins are precipitated from blood before extraction. Both blood and urine samples are extracted using solid phase extraction (SPE). Analysis of extracts is by liquid chromatography/tandem mass spectrometry in the single reaction monitoring mode (LC/MS(SRM)).

4 Specimens

This procedure uses 0.2 mL of blood (in duplicate for quantitation[1]) or 0.4 mL of urine.

5 Equipment/Materials/Reagents

a. Routine laboratory supplies, including calibrated pipettes, disposable culture tubes, test tube racks, graduated cylinders, etc.

[1] If case history, screening results, or other previous analysis indicates that the concentration of a benzodiazepine may be above the linear range of the method, the sample may be prediluted before extraction.

b. Methanol (Optima Grade)

c. Deionized water (18 MΩ)

d. 1:1 Methanol:Water

e. Zinc sulfate heptahydrate

f. Zinc Sulfate (0.2 M): Combine 50 mL deionized water and 5.75 g of zinc sulfate heptahydrate in a 100 mL volumetric flask. Mix until dissolved. Bring to the mark with deionized water. Store in glass at room temperature. Stable for at least 6 months.

g. Zinc Sulfate in Methanol: Combine 80 mL methanol and 20 mL zinc sulfate (0.2 M) in a volumetric flask and mix well. Mix well. Store in glass at room temperature. Stable for at least 2 months.

h. Potassium dihydrogen phosphate

i. Disodium hydrogen phosphate (anhydrous)

j. Potassium Phosphate Buffer: Add 9.07 g potassium dihydrogen phosphate to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.

k. Sodium Phosphate Buffer: Add 11.6 g disodium hydrogen phosphate (anhydrous) to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.

l. Sorensen Buffer (pH 7.4): Add sodium phosphate buffer to the potassium phosphate buffer until the pH reads 7.4 with a pH meter. Store refrigerated in glass or plastic. Stable for at least three months.

m. β-Glucuronidase - (>100,000 u/mL β-glucuronidase activity; from Red Abalone, ***H. Rufescena***; available from Kura Biotec)

n. Ammonium acetate (99.999% purity)

o. Acetic acid, glacial (17 M, ACS grade)

p. Ammonium Acetate Buffer (0.5 M; pH 5):
Add 3.854 g ammonium acetate to a 100-mL volumetric flask containing about 75 mL deionized water. Mix well to dissolve. Add glacial acetic acid until pH registers between 4.5 and 5.5. Bring to volume with deionized water and mix well. Store refrigerated in glass or plastic. Stable at least three months.

q. Vortexer

r. Centrifuge

s. Evaporator with nitrogen

t. SPE manifold

u. pH meter

v. Oasis HLB 6 cc (500 mg) LP SPE cartridges

w. Ammonium hydroxide, concentrated (15 M) (ACS grade)

x. Methanol:Water:Ammonia (40:60:0.5):
Combine 40 mL methanol, 60 mL deionized water and 0.5 mL ammonium hydroxide and mix well. Store at room temperature in glass. Prepare fresh daily.

y. Methylene chloride (Optima grade)

z. Isopropanol (HPLC grade)

aa. Methylene Chloride:Isopropanol (75:25):
Combine 75 mL methylene chloride and 25 mL isopropanol and mix well. Store at room temperature in glass. Stable for at least two months.

bb. Water:Acetonitrile (90:10):
Combine 90 mL deionized water and 10 mL acetonitrile (Optima grade) and mix well. Store at room temperature in glass. Stable for at least three months.

cc. Centrifuge tube filters (0.45 micron, Nylon)

dd. Ammonium formate

ee. Formic acid

ff. Mobile Phase A (5 mM Ammonium Formate with formic acid; pH~3.5): Add 0.3153 g ammonium formate to a 1 L volumetric flask. Add approximately 800 mL deionized water and mix well. Add 1 mL formic acid, and QS with deionized water. Store in glass at room temperature. Stable for at least one week.

gg. Mobile Phase B (Acetonitrile with 0.1% Formic Acid): Combine 1 mL formic acid and 1000 mL acetonitrile and mix well. Store in glass at room temperature. Stable for at least two months.

hh. ABI 5000 QTRAP Liquid Chromatograph/Mass Spectrometer equipped with Analyst software and a Phenomenex Kinetex XB-C18 (or equivalent) analytical column (150 mm x 2.1mm x 2.6 µ)

6 Standards and Controls

a. Standard and Control Stock Solutions (1.0 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed or an equivalent supplier. The materials used to prepare the standard stock solutions will be from a different source than the materials used to prepare the control stock solutions. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam	clonazepam	n-desmethylflunitrazepam
7-aminoflunitrazepam	desalkylflurazepam	nordiazepam
α-hydroxyalprazolam	diazepam	oxazepam
α-hydroxymidazolam	flunitrazepam	temazepam
α-hydroxytriazolam	flurazepam	triazolam
alprazolam	lorazepam	
chlordiazepoxide	midazolam	

b. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX) or an equivalent supplier. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam-d_4	chlordiazepoxide-d_5	midazolam-d_4
7-aminoflunitrazepam-d_7	clonazepam-d_4	n-desmethylflunitrazepam-d_4
α-hydroxyalprazolam-d_5	desalkylflurazepam-d_4	nordiazepam-d_5
α-hydroxymidazolam-d_4	diazepam-d_5	oxazepam-d_5
α-hydroxytriazolam-d_4	flunitrazepam-d_7	temazepam-d_5
alprazolam-d_5	lorazepam-d_4	triazolam-d_4
oxazepam glucuronide-d_5		

i. Internal Standard Intermediate Solution (5 µg/mL):
Add 0.25 mL of each Internal Standard Stock Solution to a 5-mL volumetric flask and bring to the mark with methanol. Store in the freezer. Stable for at least 2 years.

j. Internal Standard Working Solution (500 ng/mL):
Combine 0.1 mL of the Internal Standard Intermediate Solution (5 µg/mL) and 0.9 mL methanol. Prepare fresh daily.

k. Calibration Scheme

This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

Preparation of High Calibration Solution			
Starting Solution	1	mg/mL	stock solution(s)
Starting Solution Aliquot	0.025	mL	
Diluent Volume	25	mL	methanol, volumetric flask
Resulting Concentration	1	µg/mL	storage: freezer; stability: ≥ 1 year

Preparation of Low Calibration Solution			
Starting Solution	1	µg/mL	High Calibrator Solution
Starting Solution Aliquot	0.5	mL	
Diluent Volume	10	mL	methanol, volumetric flask
Resulting Concentration	50	ng/mL	storage: freezer; stability: ≥ 1 year

Calibrator Level	Low Cal Spike (µL)	High Cal Spike (µL)	Resulting Concentration, ng/mL (in 0.2mL of blood)
1	10		2.5
2	20		5
3	100		25
4	200		50
5		20	100
6		30	150
7		50	250
8		75	375

l. Control Scheme

Negative Control Blood is purchased from Cliniqa or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.
At least one Positive Control Blood Sample will be analyzed with each blood assay. For

quantitative analyses, both levels of Positive Control Blood Samples will be analyzed in duplicate.

Preparation of High Control Solution			
Starting Solution	1	mg/mL	stock solution(s)
Starting Solution Aliquot	0.025	mL	
Diluent Volume	25	mL	methanol, volumetric flask
Resulting Concentration	1	µg/mL	storage: freezer; stability: ≥ 1 year

Preparation of Low Control Solution			
Starting Solution	1	µg/mL	High Control Solution
Starting Solution Aliquot	0.5	mL	
Diluent Volume	10	mL	methanol, volumetric flask
Resulting Concentration	50	ng/mL	storage: freezer; stability: ≥ 1 year

Control Level	Low Control Spike (µL)	High Control Spike (µL)	Resulting Concentration, ng/mL (in 0.2mL of blood)
Negative	0	0	0
Low	20	0	5
High	0	50	250

m. Hydrolysis Check Internal Standard Intermediate Solution (2.0 µg/mL d_5-Oxazepam equivalent): To a 10-mL volumetric flask, add 0.322 mL of the d_5-Oxazepam Glucuronide Stock Standard. Bring to volume with acetonitrile. Store frozen in glass. Stable for at least 6 months.

n. Hydrolysis Check Internal Standard Working Solution (200 ng/mL d5-Oxazepam equivalent): Combine 0.1 mL of the Hydrolysis Check Internal Standard Intermediate Solution (2.0ug/mL) and 0.9 mL deionized water. Prepare fresh daily.

o. Negative Control Urine:
Prepared in-house or purchased from an appropriate vendor. Stable for 6 months when refrigerated. A Negative Control Urine sample will be extracted and analyzed with every urine assay.

p. Positive Control Urine Samples:

1. Low Positive Control Urine (1 ng/mL):
   Mix 0.020 mL of the Benzodiazepine Working Standard Control Solution (50 ng/mL) with 1.0 mL of Negative Control Urine. Mix well before withdrawing 0.4 mL for analysis. Prepare fresh.
2. High Positive Control Urine (10 ng/mL):
   Mix 0.010 mL of the Intermediate Standard Control Solution (1 µg/mL) with 1.0 mL of Negative Control Urine. Mix well before withdrawing 0.4 mL for analysis. Prepare fresh.
3. Hydrolysis Control Urine (10 ng/mL):
   Add 0.020 mL of the Hydrolysis Check Internal Standard to a 0.4 mL aliquot of the high positive control urine. (This results in a 10 ng/mL concentration of d_5-oxazepam.) Prepare fresh.

At least one Positive Control Urine Sample will be analyzed with every urine assay.

q. LC/MS Performance Standard (5 ng/mL): Add 5 µL of the Benzodiazepine Intermediate Standard Calibrator Solution (1 µg/mL) and 10 µL of the Internal Standard Working Solution to 1 mL of Water:Acetonitrile (90:10). Store in refrigerated autosampler tray for up to one week or prepare fresh daily.

r. This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows the amount of the Benzodiazepine Working Standard Calibrator Solution to add to 0.2 mL of Negative Control Blood for calibrator preparation.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

8.1 Sample Preparation for Blood Specimens

a. Prepare blood calibrators and controls as directed in Section 6 above. A second chemist will prepare the control solutions and positive control blood samples.

b. Pipet 0.2 mL of each case blood sample into a properly labeled 12 x 75 mm test tube.

(Blood samples will be prepared in duplicate for quantitation and may have to be diluted for benzodiazepine concentrations that are above the linear range of the procedure.)

c. Add 0.020 mL of the Internal Standard Working Solution (500 ng/mL) to each sample and vortex well.

d. Add 2 mL zinc sulfate in methanol to each blood sample. Allow to sit for 1 minute, then vortex.

e. Centrifuge samples for 5 minutes at 3000 rpm.

f. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube.

g. Concentrate samples to ~0.4 mL under nitrogen at 60°C.

h. Add 5.5 mL of Sorenson buffer to each tube.

i. Vortex and centrifuge samples for 1 minute at 3000 rpm.

8.2 Sample Preparation for Urine Specimens

a. Prepare urine controls as directed in Section 6 above.

b. Pipet 0.4 mL of each case urine sample into a properly labeled 16 x 100 mm test tube.

c. Add 0.010 mL of the Internal Standard Working Solution (500 ng/mL) to each sample (except the hydrolysis control sample) and vortex well.

d. Add 0.6 mL Ammonium Acetate Buffer (0.5 M, pH 5) and 0.1 mL β-glucuronidase.

e. Vortex, cap, and incubate 30 minutes at 68°C.

f. Cool to room temperature.

g. Add 2 mL zinc sulfate in methanol to each urine sample. Allow to sit for 1 minute, then vortex.

h. Centrifuge samples for 5 minutes at 3000 rpm.

i. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube.

j. Concentrate samples to ~1.0 mL under nitrogen at 60°C.

k. Add 5 mL of Sorenson buffer to each tube.

l. Vortex and centrifuge samples for 1 minute at 3000 rpm.

8.3 Solid Phase Extraction (applicable to blood and urine samples)

a. Pre-rinse SPE extraction cartridge (Oasis HLB) by adding 2 mL of methanol.

b. Condition cartridge with 3 mL of deionized water.

c. Load sample on SPE cartridge.

d. Wash cartridge with 2 mL of Methanol:Water:Ammonia (40:60:0.5).

e. Dry cartridge at full vacuum for 15 minutes. (Use vacuum manifold; positive pressure source not shown to dry effectively.

f. Elute with 5 mL Dichloromethane:Isopropanol (75:25) under gravity.

g. Evaporate eluent to dryness at 60°C under nitrogen.

h. Reconstitute blood extracts with 0.25 mL Water:Acetonitrile (90:10). Vortex. Reconstitute urine extracts with 0.1 mL of Water:Acetonitrile (90:10). Vortex.

i. Filter samples through 0.45 micron filters.

j. Analyze 5 µL of the LC/MS Performance Standard to verify that the instrument is operating properly and that retention times have not shifted outside of the analytes' multipole reaction monitoring (MRM) windows.

k. Analyze extracts following the instrumental conditions in Section 9 below.

9 Instrumental Conditions

Appendix 1 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Autosampler Parameters

a. Autosampler Temperature Setting: 14°C
b. Injection volume = 5 µL for blood; 20 µL for urine.

9.2 Liquid Chromatograph Parameters

Column Oven Temp	23°C	
Time (min)	% Mobile Phase A (Aqueous)	% Mobile Phase B (Organic)
0:01	90	10
12:00	60	40
21:00	60	40
25:00	0	100
28:00	90	10
40:00	90	10
Flow rate	0.2 mL/min	

9.3 Mass Spectral Parameters

Scan Mode	Turbo Spray	**Polarity**	Positive
Resolution	Unit	**Scan Type**	MRM
Curtain Gas	Nitrogen (35)	**Ionspray Voltage**	3000
Source Temperature	670°C	**Nebulizer Gas**	Nitrogen (50)
Interface Heater	ON	**Turbo Gas**	Nitrogen (50)
Collision Gas	Nitrogen (Medium)	**Entrance Potential**	10

Q1 Mass	Q3 Mass	Time (min)*	Declustering Potential	Collision Energy	Collision Exit Potential
309.266	281.200	16.66	76	37	14
309.266[a]	205.100	16.66	76	57	22
311.237	283.200	16.66	126	35	30
314.253	279.300	16.56	131	37	38
316.194[a]	270.200	17.24	146	35	16
316.194	214.100	17.24	146	53	16
318.184	272.200	17.24	141	37	22
320.238	274.200	17.16	191	33	18
285.086[a]	193.200	21.90	126	43	20
285.086	154.100	21.90	126	37	10
287.214	193.100	21.90	141	43	18
290.203	198.200	21.60	46	43	26
314.220[a]	268.200	18.66	106	35	16
314.220	239.200	18.66	106	47	24
314.220	183.100	18.66	106	81	20

Q1 Mass	Q3 Mass	Time (min)*	Declustering Potential	Collision Energy	Collision Exit Potential
321.295	275.300	18.48	101	37	26
388.577[a]	315.200	13.62	56	31	18
388.577	288.200	13.62	56	35	30
390.313	317.200	13.62	121	33	24
289.173[a]	226.200	17.90	36	45	22
289.173	179.100	17.90	36	61	12
289.173	165.000	17.90	36	35	20
291.132	226.200	17.90	111	41	20
293.205	230.200	17.82	56	39	24
321.203[a]	275.100	16.61	91	31	28
321.203	229.200	16.61	91	43	24
323.186	277.200	16.61	96	31	20
327.203	281.100	16.55	146	33	22
326.205	291.300	13.34	176	37	22
326.205[a]	249.100	13.34	176	51	26
326.205	222.300	13.34	176	63	18
328.190	291.200	13.34	181	37	30
271.258	165.300	17.60	141	41	0
271.258[a]	208.300	17.60	141	43	0
271.258	243.300	17.60	141	31	4
276.382	213.200	17.46	26	41	22
287.231[a]	241.300	16.01	161	31	24
287.231	231.200	16.01	161	31	24
289.213	243.200	16.01	106	31	26
292.196	246.200	15.93	101	33	16
301.266[a]	255.100	18.40	101	31	26
301.266	177.000	18.40	101	53	18
303.251	257.200	18.40	71	31	16
306.215	260.200	18.30	81	33	26
343.213	308.300	17.12	36	37	32
343.213[a]	239.200	17.12	36	53	26
345.212	241.100	17.12	36	57	36
347.216	243.300	17.04	166	57	10
325.250[a]	297.200	15.28	106	37	16
325.250	216.100	15.28	106	53	22
327.247	299.200	15.28	181	35	30
330.270	302.300	15.23	136	37	20
286.233[a]	222.200	9.40	131	35	18
286.233	250.200	9.40	131	29	14

Q1 Mass	Q3 Mass	Time (min)*	Declustering Potential	Collision Energy	Collision Exit Potential
286.233	195.200	9.40	131	43	12
288.219	222.200	9.40	111	35	26
290.218	226.200	9.32	126	33	20
284.285 [a]	227.300	10.92	151	35	16
284.285	240.300	10.92	151	45	18
284.285	163.200	10.92	151	31	16
342.211[a]	203.000	13.35	71	35	20
342.211	168.100	13.35	71	51	10
344.216	205.000	13.35	111	37	22
359.211	331.300	15.30	151	39	20
359.211[a]	176.000	15.30	151	37	26
359.211	239.200	15.30	151	61	24
361.195	333.200	15.30	151	39	34
363.229	176.000	15.26	176	39	20
300.173	254.200	16.26	91	33	54
300.173[a]	198.100	16.26	91	51	24
300.173	225.100	16.26	91	49	12
304.211	258.200	16.20	116	35	18
300.217[a]	227.100	11.64	91	35	26
300.217	247.200	11.64	91	49	20
300.217	165.100	11.64	91	67	10
305.133	232.100	11.56	81	35	24
330.237	253.100	13.30	126	55	26
346.232	203.000	13.29	151	37	22
291.285	138.000	10.80	121	41	20

*MRM Times may be adjusted over time due to changes in mobile phase and/or column performance. Small changes (less than 2 minutes) in these times are not considered modifications to the method and need not be recorded as modifications in case notes.
[a] This is the typical quant transition referred to in Section 11 below.

10 Decision Criteria

10.1 LC/MS Performance Standard Decision Criteria

Peaks should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. The chemist should ensure that the peaks entirely elute within their MRM windows, and adjust the MRM window times, if necessary.

10.2 Unknown Sample Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

10.2.1 Batch Acceptance

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected in the LC/MS data. High and Low Positive Controls should fall within ±20% of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for further guidance.

There should be a peak for d5-oxazepam in the Hydrolysis Check Positive Control Urine. This peak area should approximate the area of the oxazepam peak (within ±50%).

10.2.2 Unknown Sample Criteria

Each of the Internal Standards should be detectable in the LC/MS data.

10.2.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.2.2.2 Retention Time

The retention time of the peak should be within ± 2% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

10.2.2.3 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 10 when using the Analyst software. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

10.2.2.4 Mass Spectrometry

At least three independent MS/MS experiments are conducted for each analyte. (See Table 2 below.) Two ion ratios are calculated for each analyte. The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

Table 2: MS/MS Transitions

Analyte	Tran 1	Tran 2	Tran 3	Trans 4
Alprazolam	309.2→281.2	309.2→205.1	311.2→283.2	n/a
Clonazepam	316.1→270.2	316.1→214.1	318.1→272.2	n/a
Diazepam	285.0→193.2	285.0→154.1	287.2→193.1	n/a
Flunitrazepam	314.2→268.2	314.2→239.2	314.2→183.1	n/a
Flurazepam	388.5→315.2	388.5→288.2	390.3→317.2	n/a
Desalkylflurazepam	289.1→226.2	289.1→165.0	291.1→226.2	289.1→179.1
Lorazepam	321.2→275.1	321.2→229.2	323.1→277.2	n/a
Midazolam	326.2→291.3	326.2→249.1	326.2→222.3	328.1→291.2
Nordiazepam	271.2→165.3	271.2→208.3	271.2→243.3	n/a
Oxazepam	287.2→241.3	287.2→231.2	289.2→243.2	n/a
Temazepam	301.2→255.1	301.2→177.0	303.2→257.2	n/a
Triazolam	343.2→308.3	343.2→239.2	345.2→241.1	n/a
α-hydroxyalprazolam	325.2→297.2	325.2→216.1	327.2→299.2	n/a
7-aminoclonazepam	286.2→222.2	286.2→250.2	286.2→195.2	288.2→222.2
7-aminoflunitrazepam	284.2→227.3	284.2→240.3	284.2→163.2	n/a
α-hydroxymidazolam	342.2→203.0	342.2→268.1	344.2→205.0	n/a
α-hydroxytriazolam	359.2→331.3	359.2→176.0	359.2→239.2	361.2→333.2
desmethylflunitrazepam	300.1→254.2	300.1→198.1	300.1→225.1	n/a
chlordiazepoxide	300.2→227.1	300.2→247.2	300.2→165.1	n/a

11 Calculations

$1/x^2$ weighting is used for all calibration curves. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory Report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

13 Limitations

a. Limit of Detection:
   1. Blood: 1.25 ng/mL (or lower)
   2. Urine: 0.5 ng/mL (or lower)

b. Limit of Quantitation: 2.5 ng/mL

c. Accuracy (as % bias; n=15 for all values in table):

	Bias (%; at 5 ng/mL)	Bias (%; at 100 ng/mL)	Bias (%; at 250 ng/mL)
7-aminoclonazepam	-3.63	2.06	3.65
7-aminoflunitrazepam	2.71	5.89	1.32
α-OH midazolam	3.93	3.76	0.44
α-OH alprazolam	3.83	3.88	-1.22
diazepam	5.21	4.54	-2.80
clonazepam	0.39	3.69	1.41
alprazolam	6.84	6.45	-1.85
chlordiazepoxide	0.40	2.11	-2.69
flunitrazepam	1.23	2.09	-0.17
desalkylflurazepam	2.71	2.42	0.36
lorazepam	2.04	2.89	0.69
flurazepam	2.89	-1.79	-0.79
n-desmethylflunitrazepam	0.09	2.11	-0.78
midazolam	0.03	2.17	1.01
nordiazepam	2.29	4.72	-2.76
oxazepam	0.52	0.97	-1.00
temazepam	5.29	5.26	-2.21
triazolam	7.57	5.43	-3.56

α-OH triazolam	2.31	4.94	-0.14

d. Precision (as both repeatability and intermediate precision; n=15 for all values in tables):

	Repeatability (%; at 5 ng/mL)	Repeatability (%; at 100 ng/mL)	Repeatability (%; at 250 ng/mL)
7-aminoclonazepam	7.20	9.71	8.96
7-aminoflunitrazepam	7.29	6.49	11.09
α-OH midazolam	5.45	3.22	4.26
α-OH alprazolam	4.95	2.78	4.22
diazepam	5.26	2.34	3.33
clonazepam	4.72	2.47	3.86
alprazolam	4.83	3.66	2.85
chlordiazepoxide	3.70	3.14	4.38
flunitrazepam	4.54	3.02	2.76
desalkylflurazepam	4.79	4.03	4.01
lorazepam	6.79	3.28	3.12
flurazepam	9.55	5.69	3.70
n-desmethylflunitrazepam	3.94	3.62	3.76
midazolam	4.31	3.17	3.75
nordiazepam	5.00	2.79	3.73
oxazepam	5.08	2.65	3.08
temazepam	4.32	2.70	2.96
triazolam	4.59	3.17	1.92
α-OH triazolam	4.67	3.37	4.00

	Intermediate Precision (%; at 5 ng/mL)	Intermediate Precision (%; at 100 ng/mL)	Intermediate Precision (%; at 250 ng/mL)
7-aminoclonazepam	10.29	9.71	9.17
7-aminoflunitrazepam	7.29	6.49	11.65
α-OH midazolam	6.45	5.25	5.74
α-OH alprazolam	5.72	5.04	5.37
diazepam	5.36	3.94	5.95
clonazepam	4.91	4.05	5.37
alprazolam	5.63	5.04	6.66
chlordiazepoxide	4.45	5.04	6.63
flunitrazepam	5.04	4.74	4.47
desalkylflurazepam	5.44	4.64	6.60
lorazepam	7.67	7.38	7.53
flurazepam	10.45	7.50	6.29
n-desmethylflunitrazepam	5.40	4.50	4.81
midazolam	5.29	5.09	6.33
nordiazepam	5.40	5.20	5.40
oxazepam	5.68	3.29	3.16
temazepam	5.52	5.84	5.45

triazolam	5.11	5.21	5.34
α-OH triazolam	4.91	5.83	6.22

e. Cautionary Statement: Oxazepam may be unstable in methanol.

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 9th ed.; Biomedical Publications, Seal Beach, California, 2011.

Guidelines for Toxicological Quantitations (Tox 101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

Chemistry Unit Procedures for Estimating Measurement Uncertainty (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

FBI Laboratory Safety Manual.

Rev. #	Issue Date	History
5	02/09/18	Updated Scope language. Updated calibration/control scheme preparation in Section 6. Removed "reasonable degree of scientific certainty" language from Section 10.2.2.4. Updated approval lines. Removed TOX103 reference from Section 15. In Section 8.3e, specified vacuum manifold vs positive pressure. In section 6, reformatted the Calibration and Control scheme into a tabular format, and updated to a simpler preparation (also updated on the bench notes, which made a total of 3 pages from 2 pages).
6	04/01/19	Removed "subunit" (header, 15). Updated CUQA title in Reference Section and 12. Updated blood reconstitution volume to 0.25mL (8.3-h, Appendix). Made format/typo corrections on page 1 of Appendix. Updated enzyme to >100,000 (5-m) to account for product description.

Approval

Redacted - Signatures on File

Toxicology Technical Leader: Date: 03/28/2019

Chemistry Unit Chief: Date: 03/28/2019

QA Approval

Quality Manager: Date: 03/28/2019

Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use (page 1-3)

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